REMARKS

Claim 1 is amended to correct a typographical error introduced by the previous amendment. It should be noted that this amendment is not narrowing. The pending claims and the amendment is clearly supported throughout the specification and the original claims. No new matter is believed to be introduced by the amendment.

Claims 1-20 are pending. Favorable reconsideration is respectfully requested.

The rejections of Claims 1-20 35 U.S.C. § 102 and/or § 103 over Kim et al. (Applied ad Environmental Microbiology) published March, 1999 is believed to be obviated by the perfection of priority under 37 C.F.R. §1.55. A copy of the Certified English-language translation of the priority document is enclosed.

Applicants submit herewith the certified English language translation of the foreign priority application (JP JP 11-50562, filed on February 26, 1999). Since the filing date of the foreign priority application is earlier than the publication date of Kim et al. (Applied ad Environmental Microbiology) published March, 1999, the rejection of Claims 1-20 under 35 U.S.C. §102(a) and/or §103 over Kim et al. (Applied ad Environmental Microbiology) published March, 1999 is obviated. Accordingly, withdrawal of these ground of rejection is respectfully requested.

The rejection of Claims 1-20 under 35 U.S.C. § 102 and/or § 103 over Kim et al. (Journal of Fermentation and Bioengineering) is traversed below.

Kim et al. (Journal of Fermentation and Bioengineering) discloses, at best, an isolated **microorganism**, Geotricum candidum Dec1, which may decolorize eighteen dyes and three model compounds (See Abstract and Table 2 of this reference). The Office has taken the position that the "broad teachings of Kim et al. appear to anticipate the material limitations of

the instant claims." However, Applicants have not been directed to where the specific disclosures occur within Kim et al. that state all of the claim limitations. More specifically, the Office has not provided Appicants with the the portions of the cited reference that actually disclose the claimed invention. There is merely a hand-waiving blanket placed on the reference as "broad teachings". Further, how can a reference "appear" to anticipate the claimed invention? It appears to the Applicants, as if the Office is placing the cart before the horse in establishing both anticipation and/or obviousness in light of its own position in the Office Action.

In direct contrast to Kim et al., the present invention relates to **an isolated peroxidase enzyme (DyP)**, not a microorganism, having the following characteristics:

a) a property to degrade and decolorize a dye;

b) a molecular weight of 60 kDa, by the molecular weight assay as determined by SDS-PAGE;

c) a molecular weight of 55 kDa, by the molecular weight assay as determined by gel filtration; and

d) pI 3.8, as determined by an assay of isoelectric focusing.

Applicants respectfully traverse the outstanding rejection on the basis that in no way can a disclosed **microorganism** anticipate and/or even suggest an **isolated enzyme** absent some very specific explanation on the part of the Office. These are two completely different chemical entities. Further, it would be impossible for a microorganism to have all the above-mentioned properties that characterize the claimed isolated enzyme. It is not the Applicants' burden to overcome a case of anticipation and/or obviousness that may "appear" to exist in the absence of concrete evidence of the same. Accordingly, Kim et al. must anticipate or must not anticipate the claimed invention. The reference can not "appear to anticipate" the

claimed invention. Since Kim et al. relates to a microorganism and the claimed invention relates, in part, to an enzyme, Kim et al. can not possible anticipate the claimed invention.

In light of the above, it appears as if the Office is relying on the Applicants disclosure to supply motivation to modify the disclosed of Kim et al. to the claimed enzyme. However, this simply can not be possible because Kim et al., at best, discloses a microorganizm that cannot possible satisfy the above-mentioned claimed characteristics (a) - (d) of the claimed enzyme. Moreover, this is clearly improper procedure according to a recent decision by the U.S. Federal Courts in *In re Lee* (61 USPQ2d 1430), copy provided herewith.

The *Lee* Court indicated that the Office must provide specific motivation, hint, or suggestion, found in the references relied upon to support a *prima facia* case of obviousness. In the present case, the Office appears to rely on the present specification for motivation, which is clearly forbidden according to the *Lee* Court. In light of this decision, Applicants respectfully request the Office not to use the present specification to find motivation that is not present in any of the disparate disclosure of the reference discussed herein. Accordingly, withdrawal of this ground of rejection is respectfully requested.

Applicants respectfully submit that the present application is now in condition for allowance. Favorable reconsideration is respectfully requested. Should anything further be required to place the application in condition for allowance, the Examiner is requested to contact the undersigned by telephone.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Norman F. Oblon
Attorney of Record
Registration No. 24,618

Thomas W. Barnes III, Ph.D.
Registration No. 52,595

22850

Tel.: (703) 413-3000
Fax: (703) 413-2220
NFO:TWB\smi

Part 8 # 16

61 USPQ2D 1430
In re Lee

U.S. Court of Appeals Federal Circuit

No. 00-1158
Decided January 18, 2002

Headnotes

PATENTS

[1] Practice and procedure in Patent and Trademark Office —Board of Patent Appeals and Interferences — In general (§110.1101)

Patentability/Validity — Obviousness — Combining references (§115.0905)

Patentability/Validity — Obviousness — Evidence of (§115.0906)

Rejection of patent application for obviousness under 35 U.S.C. §103 must be based on evidence comprehended by language of that section, and search for and analysis of prior art includes evidence relevant to finding of whether there is teaching, motivation, or suggestion to select and combine references relied on as evidence of obviousness; factual inquiry whether to combine references must be thorough and searching, based on objective evidence of record, and Board of Patent Appeals and Interferences must explain reasons why one of ordinary skill in art would have been motivated to select references and to combine them to render claimed invention obvious.

[2] Patentability/Validity — Obviousness — Combining references (§115.0905)

JUDICIAL PRACTICE AND PROCEDURE

Procedure — Judicial review — Standard of review —Patents (§410.4607.09)

Board of Patent Appeals and Interferences improperly relied upon "common knowledge and common sense" of person of ordinary skill in art to find invention of patent application obvious over combination of two prior art references, since factual question of motivation to select and combine references is material to patentability, and could not be resolved on subjective belief and unknown authority, since deferential review of agency decisions under Administrative Procedure Act reinforces obligation of board to develop evidentiary basis for its findings, since board's rejection of need for any specific hint or suggestion in particular reference to support combination constituted omission of relevant factor required by precedent, and thus was both legal error and arbitrary agency action, since board's findings must extend to all material facts and be documented on record, and since "common knowledge and common sense" are not specialized knowledge and expertise of agency contemplated by APA, and may not be substituted for evidence, although they may be applied to analysis of evidence.

PATENTS

[3] Practice and procedure in Patent and Trademark Office —Board of Patent Appeals and Interferences — In general (§110.1101)

Patentability/Validity — Obviousness — Evidence of (§115.0906)

JUDICIAL PRACTICE AND PROCEDURE

Procedure — Judicial review — Standard of review —Patents (§410.4607.09)

Patent examiners and Board of Patent Appeals and Interferences, in relying on what they assert to be general knowledge to negate patentability on ground of obviousness, must articulate that knowledge and place it on record, since examiners and board are presumed

to act from viewpoint of person of ordinary skill in art in finding relevant facts, assessing significance of prior art, and making ultimate determination of obviousness issue; failure to do so is not consistent with either effective administrative procedure or effective judicial review, and

board cannot rely on conclusory statements when dealing with particular combinations of prior art and specific claims, but must set forth rationale on which it relies.

[4] Procedure — Court of Appeals for the Federal Circuit (§410.03)

Procedure — Judicial review — Standard of review —Patents (§410.4607.09)

U.S. Court of Appeals for the Federal Circuit will not consider proposed alternative grounds for affirming decision of Board of Patent Appeals and Interferences rejecting patent application for obviousness, since alternative grounds were made at oral argument and constitute post hoc rationalization for agency action, consideration of which would deprive aggrieved party of fair opportunity to support its position.

Case History and Disposition

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent application of Sang-Su Lee, serial no. 07/631,210, directed to method of automatically displaying functions of video display device and demonstrating how to select and adjust functions to facilitate user response. Applicant appeals from decision upholding rejection of all claims for obviousness, and from reaffirmation of that decision on reconsideration. Reversed and remanded.

Attorneys:

Richard H. Stern and Robert E. Bushnell, Washington, D.C., for Sang Su Lee.

Sidney O. Johnson Jr., associate solicitor, John M. Whealan, solicitor, and Raymond T. Chen, Maximilian R. Peterson, and Mark Nagumo, associate solicitors, Arlington, Va., for Director of U.S. Patent and Trademark Office.

Judge:

Before Newman, Clevenger, and Dyk, circuit judges.

Opinion Text

Opinion By:

Newman, J.

Sang-Su Lee appeals the decision of the Board of Patent Appeals and Interferences of the United States Patent and Trademark Office, rejecting all of the claims of Lee's patent application Serial No. 07/631,210 entitled "Self-Diagnosis and Sequential-Display Method of Every

Function." 1We vacate the Board's decision for failure to meet the adjudicative standards for review under the Administrative Procedure Act, and remand for further proceedings.

The Prosecution Record

Mr. Lee's patent application is directed to a method of automatically displaying the functions of a video display device and demonstrating how to select and adjust the functions in order to facilitate response by the user. The display and demonstration are achieved using computer-managed electronics, including pulse-width modulation and auto-fine-tuning pulses, in accordance with procedures described in the specification. Claim 10 is representative:

> 10. A method for automatically displaying functions of a video display device, comprising:
> determining if a demonstration mode is selected;
> if said demonstration mode is selected, automatically entering a picture adjustment mode having a picture menu screen displaying a list of a plurality of picture functions; and
> automatically demonstrating selection and adjustment of individual ones of said plurality of picture functions. The examiner rejected the claims on the ground of obviousness, citing the combination of two references: United States Patent No. 4,626,892 to Nortrup, and the Thunderchopper Helicopter Operations Handbook for a video game. The Nortrup reference describes a television set having a menu display by which the user can adjust various picture and audio functions; however, the Nortrup display does not include a demonstration of how to adjust the functions. The Thunderchopper Handbook describes the Thunderchopper game's video display as having a "demonstration mode" showing how to play the game; however, the Thunderchopper Handbook makes no mention of the adjustment of picture or audio functions. The examiner held that it

> would have been obvious to a person of ordinary skill to combine the teachings of these references to produce the Lee system.

Lee appealed to the Board, arguing that the Thunderchopper Handbook simply explained how to play the Thunderchopper game, and that the prior art provided no teaching or motivation or suggestion to combine this reference with Nortrup, or that such combination would produce the Lee invention. The Board held that it was not necessary to present a source of a teaching, suggestion, or motivation to combine these references or their teachings. The Board stated:

> The conclusion of obviousness may be made from common knowledge and common sense of a person of ordinary skill in the art without any specific hint or suggestion in a particular reference. Board op. at 7. The Board did not explain the "common knowledge and common sense" on which it relied for its conclusion that "the combined teachings of Nortrup and Thunderchopper would have suggested the claimed invention to those of ordinary skill in the art."

Lee filed a request for reconsideration, to which the Board responded after five years. The

Board reaffirmed its decision, stating that the Thunderchopper Handbook was "analogous art" because it was "from the same field of endeavor" as the Lee invention, and that the field of video games was "reasonably pertinent" to the problem of adjusting display functions because the Thunderchopper Handbook showed video demonstrations of the "features" of the game. On the matter of motivation to combine the Nortrup and Thunderchopper references, the Board stated that "we maintain the position that we stated in our prior decision" and that the Examiner's Answer provided "a well reasoned discussion of why there is sufficient motivation to combine the references." The Board did not state the examiner's reasoning, and review of the Examiner's Answer reveals that the examiner merely stated that both the Nortrup function menu and the Thunderchopper demonstration mode are program features and that the Thunderchopper mode "is user-friendly" and it functions as a tutorial, and that it would have been obvious to combine them.

Lee had pressed the examiner during prosecution for some teaching, suggestion, or motivation in the prior art to select and combine the references that were relied on to show obviousness. The Examiner's Answer before the Board, plus a Supplemental Answer, stated that the combination of Thunderchopper with Nortrup "would have been obvious to one of ordinary skill in the art since the demonstration mode is just a programmable feature which can be used in many different device[s] for providing automatic introduction by adding the proper programming software," and that "another motivation would be that the automatic demonstration mode is user friendly and it functions as a tutorial." The Board adopted the examiner's answer, stating "the examiner has provided a well reasoned discussion of these references and how the combination of these references meets the claim limitations." However, perhaps recognizing that the examiner had provided insufficient justification to support combining the Nortrup and Thunderchopper references, the Board held, as stated *supra*, that a "specific hint or suggestion" of motivation to combine was not required.

This appeal followed.

Judicial Review

Tribunals of the PTO are governed by the Administrative Procedure Act, and their rulings receive the same judicial deference as do tribunals of other administrative agencies. *Dickinson v. Zurko*, 527 U.S. 150, 50 USPQ2d 1930 (1999). Thus on appeal we review a PTO Board's findings and conclusions in accordance with the following criteria:

> 5 U.S.C. §706(2) The reviewing court shall—
> (2) hold unlawful and set aside agency actions, findings, and conclusions found to be—
> (A) arbitrary, capricious, an abuse of discretion, or otherwise not in accordance with law;
> * * * *
> (E) unsupported by substantial evidence in a case subject to sections 556 and 557 of this title or otherwise reviewed on the record of an agency hearing provided by statute;

For judicial review to be meaningfully achieved within these strictures, the agency tribunal

must present a full and reasoned explanation of its decision. The agency tribunal

must set forth its findings and the grounds thereof, as supported by the agency record, and explain its application of the law to the found facts. The Court has often explained:

> The Administrative Procedure Act, which governs the proceedings of administrative agencies and related judicial review, establishes a scheme of "reasoned decisionmaking." Not only must an agency's decreed result be within the scope of its lawful authority, but the process by which it reaches that result must be logical and rational. *Allentown Mack Sales and Service, Inc. v. National Labor Relations Bd.*, 522 U.S. 359, 374 (1998) (citation omitted). This standard requires that the agency not only have reached a sound decision, but have articulated the reasons for that decision. The reviewing court is thus enabled to perform meaningful review within the strictures of the APA, for the court will have a basis on which to determine "whether the decision was based on the relevant factors and whether there has been a clear error of judgment." *Citizens to Preserve Overton Park v. Volpe*, 401 U.S. 402, 416 (1971). Judicial review of a Board decision denying an application for patent is thus founded on the obligation of the agency to make the necessary findings and to provide an administrative record showing the evidence on which the findings are based, accompanied by the agency's reasoning in reaching its conclusions. *See In re Zurko*, 258 F.3d 1379, 1386, 59 USPQ2d 1693, 1697(Fed. Cir. 2001) (review is on the administrative record); *In re Gartside*, 203 F.3d 1305, 1314, 53 USPQ2d 1769, 1774(Fed. Cir. 2000) (Board decision "must be justified within the four corners of the record").

[1] As applied to the determination of patentability *vel non* when the issue is obviousness, "it is fundamental that rejections under 35 U.S.C. §103 must be based on evidence comprehended by the language of that section." *In re Grasselli*, 713 F.2d 731, 739, 218 USPQ 769, 775(Fed. Cir. 1983). The essential factual evidence on the issue of obviousness is set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 17-18, 148 USPQ 459, 467 (1966) and extensive ensuing precedent. The patent examination process centers on prior art and the analysis thereof. When patentability turns on the question of obviousness, the search for and analysis of the prior art includes evidence relevant to the finding of whether there is a teaching, motivation, or suggestion to select and combine the references relied on as evidence of obviousness. *See, e.g., McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1351-52, 60 USPQ2d 1001, 1008(Fed. Cir. 2001) ("the central question is whether there is reason to combine [the] references," a question of fact drawing on the *Graham* factors).

"The factual inquiry whether to combine references must be thorough and searching." *Id.* It must be based on objective evidence of record. This precedent has been reinforced in myriad decisions, and cannot be dispensed with. *See, e.g., Brown & Williamson Tobacco Corp. v. Philip Morris Inc.*, 229 F.3d 1120, 1124-25, 56 USPQ2d 1456, 1459 (Fed. Cir. 2000) ("a showing of a

suggestion, teaching, or motivation to combine the prior art references is an `essential component of an obviousness holding'") (quoting *C.R. Bard, Inc., v. M3 Systems, Inc.*, 157 F.3d 1340, 1352, 48 USPQ2d 1225, 1232(Fed. Cir. 1998)); *In re Dembiczak*, 175 F.3d 994, 999, 50 USPQ2d 1614, 1617(Fed. Cir. 1999) ("Our case law makes clear that the best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for a showing of the teaching or motivation to combine prior art references."); *In re Dance*, 160 F.3d 1339, 1343, 48 USPQ2d 1635, 1637(Fed. Cir. 1998) (there must be some motivation, suggestion, or teaching of the desirability of making the specific combination that was made by the applicant); *In re Fine*, 837 F.2d 1071, 1075, 5 USPQ2d 1596, 1600(Fed. Cir. 1988) ("`teachings of references can be combined *only* if there is some suggestion or incentive to do so.'") (emphasis in original) (quoting *ACS Hosp. Sys., Inc. v. Montefiore Hosp.*, 732 F.2d 1572, 1577, 221 USPQ 929, 933(Fed. Cir. 1984)).

The need for specificity pervades this authority. *See, e.g., In re Kotzab*, 217 F.3d 1365, 1371, 55 USPQ2d 1313, 1317(Fed. Cir. 2000) ("particular findings must be made as to the reason the skilled artisan, with no knowledge of the claimed invention, would have selected these components for combination in the manner claimed"); *In re Rouffet*, 149 F.3d 1350, 1359, 47 USPQ2d 1453, 1459(Fed. Cir. 1998) ("even when the level of skill in the art is high, the Board must identify specifically the principle, known to one of ordinary skill, that suggests the claimed combination.

In other words, the Board must explain the reasons one of ordinary skill in the art would have been motivated to select the references and to combine them to render the claimed invention obvious."); *In re Fritch*, 972 F.2d 1260, 1265, 23 USPQ2d 1780, 1783(Fed. Cir. 1992) (the examiner can satisfy the burden of showing obviousness of the combination "only by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references").

[2] With respect to Lee's application, neither the examiner nor the Board adequately supported the selection and combination of the Nortrup and Thunderchopper references to render obvious that which Lee described. The examiner's conclusory statements that "the demonstration mode is just a programmable feature which can be used in many different device[s] for providing automatic introduction by adding the proper programming software" and that "another motivation would be that the automatic demonstration mode is user friendly and it functions as a tutorial"do not adequately address the issue of motivation to combine. This factual question of motivation is material to patentability, and could not be resolved on subjective belief and unknown authority. It is improper, in determining whether a person of ordinary skill would have been led to this combination of references, simply to "[use] that which the inventor taught against

its teacher." *W.L. Gore v. Garlock, Inc.*, 721 F.2d 1540, 1553, 220 USPQ 303, 312-13 (Fed. Cir. 1983). Thus the Board must not only assure that the requisite findings are made, based on evidence of record, but must also explain the reasoning by which the findings are deemed to support the agency's conclusion.

Deferential judicial review under the Administrative Procedure Act does not relieve the agency of its obligation to develop an evidentiary basis for its findings. To the contrary, the Administrative Procedure Act reinforces this obligation. *See, e.g., Motor Vehicle Manufacturers Ass'n v. State Farm Mutual Automobile Ins. Co.*, 463 U.S. 29, 43 (1983) ("the agency must examine the relevant data and articulate a satisfactory explanation for its action including a 'rational connection between the facts found and the choice made.'") (quoting *Burlington Truck Lines v. United States*, 371 U.S. 156, 168 (1962)); *Securities & Exchange Comm'n v. Chenery Corp.*, 318 U.S. 80, 94 (1943) ("The orderly function of the process of review requires that the grounds upon which the administrative agency acted are clearly disclosed and adequately sustained.").

In its decision on Lee's patent application, the Board rejected the need for "any specific hint or suggestion in a particular reference" to support the combination of the Nortrup and Thunderchopper references. Omission of a relevant factor required by precedent is both legal error and arbitrary agency action. *See Motor Vehicle Manufacturers*, 463 U.S. at 43 ("an agency rule would be arbitrary and capricious if the agency ... entirely failed to consider an important aspect of the problem"); *Mullins v. Department of Energy*, 50 F.3d 990, 992 (Fed. Cir. 1995) ("It is well established that agencies have a duty to provide reviewing courts with a sufficient explanation for their decisions so that those decisions may be judged against the relevant statutory standards, and that failure to provide such an explanation is grounds for striking down the action."). As discussed in *National Labor Relations Bd. v. Ashkenazy Property Mgt. Corp.*, 817 F.2d 74, 75 (9th Cir. 1987), an agency is "not free to refuse to follow circuit precedent."

The foundation of the principle of judicial deference to the rulings of agency tribunals is that the tribunal has specialized knowledge and expertise, such that when reasoned findings are made, a reviewing court may confidently defer to the agency's application of its knowledge in its area of expertise. Reasoned findings are critical to the performance of agency functions and judicial reliance on agency competence. *See Baltimore and Ohio R. R. Co. v. Aberdeen & Rockfish R. R. Co.*, 393 U.S. 87, 91-92 (1968) (absent reasoned findings based on substantial evidence effective review would become lost "in the haze of so-called expertise"). The "common knowledge and common sense" on which the Board relied in rejecting Lee's application are not the specialized knowledge and expertise contemplated by the Administrative Procedure Act. Conclusory statements such as those here provided do not fulfill the agency's obligation. This court explained in *Zurko*, 258 F.3d at 1385, 59 USPQ2d at 1697, that "deficiencies of the cited references cannot be remedied by

the Board's general conclusions about what is `basic knowledge' or `common sense.'" The Board's findings must extend to all material facts and must be documented on the record, lest the "haze of so-called expertise" acquire insulation from accountability. "Common knowledge and common sense," even if assumed to derive from the agency's expertise, do not substitute for authority when the law requires authority. *See Allentown Mack*, 522 U.S. at 376 ("Because reasoned decisionmaking demands it, and because the systemic consequences of any other approach are unacceptable, the Board must be required to apply in fact the clearly understood legal standards that it enunciates in principle ....")

The case on which the Board relies for its departure from precedent, *In re Bozek*, 416 F.2d 1385, 163 USPQ 545 (CCPA 1969), indeed mentions "common knowledge and common sense," the CCPA stating that the phrase was used by the Solicitor to support the Board's conclusion of obviousness based on evidence in the prior art. *Bozek* did not hold that common knowledge and common sense are a substitute for evidence, but only that they may be applied to analysis of the evidence. *Bozek* did not hold that objective analysis, proper authority, and reasoned findings can be omitted from Board decisions. Nor does *Bozek*, after thirty-two years of isolation, outweigh the dozens of rulings of the Federal Circuit and the Court of Customs and Patent Appeals that determination of patentability must be based on evidence. This court has remarked, in *Smiths Industries Medical Systems, Inc. v. Vital Signs, Inc.*, 183 F.3d 1347, 1356, 51 USPQ2d 1415, 1421(Fed. Cir. 1999), that *Bozek*'s reference to common knowledge "does not in and of itself make it so" absent evidence of such knowledge.

[3] The determination of patentability on the ground of unobviousness is ultimately one of judgment. In furtherance of the judgmental process, the patent examination procedure serves both to find, and to place on the official record, that which has been considered with respect to patentability. The patent examiner and the Board are deemed to have experience in the field of the invention; however, this experience, insofar as applied to the determination of patentability, must be applied from the viewpoint of "the person having ordinary skill in the art to which said subject matter pertains,"the words of section 103. In finding the relevant facts, in assessing the significance of the prior art, and in making the ultimate determination of the issue of obviousness, the examiner and the Board are presumed to act from this viewpoint. Thus when they rely on what they assert to be general knowledge to negate patentability, that knowledge must be articulated and placed on the record. The failure to do so is not consistent with either effective administrative procedure or effective judicial review. The board cannot rely on conclusory statements when dealing with particular combinations of prior art and specific claims, but must set forth the rationale on which it relies.

Alternative Grounds

[4] At oral argument the PTO Solicitor proposed alternative grounds on which this court might

affirm the Board's decision. However, as stated in *Burlington Truck Lines, Inc. v. United States*, 371 U.S. 156, 168 (1962), "courts may not accept appellate counsel's *post hoc* rationalization for agency action." Consideration by the appellate tribunal of new agency justifications deprives the aggrieved party of a fair opportunity to support its position; thus review of an administrative decision must be made on the grounds relied on by the agency. "If those grounds are inadequate or improper, the court is powerless to affirm the administrative action by substituting what it considers to be a more adequate or proper basis." *Securities & Exchange Comm'n v. Chenery Corp.*, 332 U.S. 194, 196 (1947). As reiterated in *Federal Election Comm'n v. Akins*, 524 U.S. 11, 25 (1998), "If a reviewing court agrees that the agency misinterpreted the law, it will set aside the agency's action and remand the case — even though the agency (like a new jury after a mistrial) might later, in the exercise of its lawful discretion, reach the same result for a different reason." Thus we decline to consider alternative grounds that might support the Board's decision.

Further Proceedings

Sound administrative procedure requires that the agency apply the law in accordance with statute and precedent. The agency tribunal must make findings of relevant facts, and present its reasoning in sufficient detail that the court may conduct meaningful review of the agency action. In *Radio-Television News Directors Ass'n v. FCC*, 184 F.3d 872 (D.C.

Cir. 1999) the court discussed the "fine line between agency reasoning that is `so crippled as to be unlawful'and action that is potentially lawful but insufficiently or inappropriately explained," quoting from *Checkosky v. Securities &Exch. Comm'n*, 23 F.3d 452, 464 (D.C. Cir. 1994); the court explained that "[i]n the former circumstance, the court's practice is to vacate the agency's order, while in the latter the court frequently remands for further explanation (including discussion of the relevant factors and precedents) while withholding judgment on the lawfulness of the agency's proposed action." *Id.* at 888. In this case the Board's analysis of the Lee invention does not comport with either the legal requirements for determination of obviousness or with the requirements of the Administrative Procedure Act that the agency tribunal set forth the findings and explanations needed for "reasoned decisionmaking."Remand for these purposes is required. *See Overton Park*, 401 U.S. at 420-221 (remanding for further proceedings appropriate to the administrative process).

VACATED AND REMANDED

Footnotes

1 *Ex parte Lee*, No. 1994-1989 (Bd. Pat. App. & Int. Aug. 30, 1994; on reconsid'n Sept. 29, 1999).

- End of Case -
A0A5D6V4M8

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DECLARATION OF ACCURACY OF TRANSLATION
IN LIEU OF SWORN TRANSLATION (37 C.F.R. 1.55 6 1.68)

The undersigned translator, having an office at E-1 Bldg., 3-12, Nihombashi 3-chome, Chuo-ku, Tokyo, Japan certifies and declares that:

(1) I am fully conversant both with the Japanese and English languages.

(2) I have carefully compared the attached English language translation of Japanese Patent application Number 11-50562 filed on February 26, 1999 with the original Japanese-language patent application.

(3) The translation is, to the best of my knowledge, and belief, an accurate translation from the original into the English language.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the matter with which this translation is used.

Date: September 5, 2003

Hiroya Yano

PATENT OFFICE
JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this office.

Date of Application: February 26,1999

Application Number: 050562/1999 (Hei.11-050562)

Applicant: Meiji Seika Kaisha Ltd.

February 18, 2000
Takahiko Kondo
Commissioner,
Patent Office

Certification Number
Hei. 2000-3008385

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[Reference Number]
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[Address]
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C12N 9/11, C12N 15/00, C02F 3/34 and C11D 7/54
[Inventor]
[Address] Roze Ichigao 406, 1062-1, Ichigao-cho, Aoba-ku, Yokohama-shi, Kanagawa
[Name] Makoto Shoda
[Inventor]
[Address] 3-17-3, Shinoharahigashi, Kouhoku-ku, Yokohama-shi, Kanagawa
[Name] Yasushi Sugano
[Inventor]
[Address] c/o Bioscience Labs., Meiji Seika Kaisha Ltd., 3-1, Chiyoda 5-chome, Sakado-shi, Saitama
[Name] Hidetoshi Kubota
[Applicant]
[Identification Number] 000006091
[Name] Meiji Seika Kaisha Ltd.
[Agent]
[Identification Number] 100074077
[Patent Attorney]
[Name] Fujio Kubota
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[Proof] Requested

[Name of document] Specification

[Title of the invention] Novel enzyme with decolorizing activity and method for decolorizing dyes by using the same

[Claims]

[Claim 1] A peroxidase enzyme derived from Geotrichum candidum Dec 1 (FERM P-15348), which has the following properties:

a) a property to degrade and decolorize dyes;

b) a molecular weight of 60 kDa, by the molecular weight assay using SDS-PAGE;

c) a molecular weight of 55 kDa, by the molecular weight assay using gel filtration; and

d) pI (isoelectric point) 3.8, by the assay by isoelectric focusing.

[Claim 2] An enzyme according to claim 1, having the amino acid sequence of SQ ID NO. 7 in the sequence listing.

[Claim 3] A gene encoding the enzyme according to claim 1, having the DNA sequence of SQ ID NO. 8 in the sequence listing.

[Claim 4] An expression plasmid vector comprising the coding gene according to claim 3.

[Claim 5] A microorganism (FERM BP-7032) transfected with the expression plasmid vector according to claim 4.

[Claim 6] A method for degrading and decolorizing dyes, comprising using an enzyme according to claim 1 or a microorganism according to claim 5 for degrading and

decolorizing dyes.

[Detailed description of the invention]

[Field of the invention]

The present invention relates to a novel peroxidase enzyme with high dye degradation activity, the genetic information thereof and a method for degrading and decolorizing dyes by using the same.

[Prior art]

Many of various synthetic dyes discharged from the processes of staining fiber products and from dyestuff production processes are slightly biodegradable substances, involving much difficulty in the degradation thereof in the nature. Because such colored wastewater is hazardous for the nature, regulations over the wastewater have increasingly been tightened.

In the fields of staining industry and dyestuff production industry, wastewater containing dyes has conventionally been treated, mainly by physical or chemical methods such as adsorption, concentration, chemical transformation and incineration. Although these treatment methods are efficient, these methods disadvantageously involve secondary pollution due to the generation of hazardous byproducts and the discharge of the greenhouse effect gas via high-level energy consumption.

Recently, attention has been focused on a treatment method actively utilizing biotechnology with microorganisms or enzymes, as an alternative of the treatment methods. Several microbial strains capable of degrading dyes and colored substances have already been reported. For example, Phanerochaete chrysosporium as one species of white rot fungus is listed, which is known as one of lignin-degrading fungus.

However, all the dye-degrading microorganisms known so far have an activity to degrade only one or several types of dyes, so the ability of the microorganisms to treat dyes via degradation is naturally limited. Therefore, the development of an efficient method for treating of wastewater containing dyes has been desired.

Some of the present inventors have isolated a microorganism capable of degrading azo type- and anthraquinone type- dyes, namely Geotrichum candidum Dec 1 (FERM P-15348) from the nature and have developed a method for degrading and decolorizing a wider range of dyes by microbial treatment (Japanese Patent Laid-open No. 9-173051).

It has been assumed that the excellent ability of Geotrichum candidum Dec 1 strain to degrade dyes may possibly be based on the peroxidase activity of the fungal strain, but no instance of specific isolation or identification of such enzyme has been found. Hence, the genetic information thereof has absolutely never been elucidated.

[Problem to be solved by the invention]

The present invention has been attained toward the industrial demand as mentioned above. An object of the invention is to provide an enzyme applicable to more efficient treatment of wastewater containing dyes and a method for degrading and decolorizing dyes by using the enzyme.

The Geotrichum candidum Dec 1 strain exerts an activity to degrade a wide range of dyes and has also prominent enzyme stability. Therefore, the fungal strain per se or after immobilization on an appropriate carrier can be used for degrading dyes.

So as to enhance the industrial applicability, however, the treatment of wastewater containing dyes, particularly dye degradation should essentially be attained in an efficient manner economically.

It is useful for that purpose to use a dye-degrading enzyme owned by said microorganism through isolation and purification rather than to use the microorganism per se, to further elucidate the genetic constitution thereof to realize the mass production of the enzyme and to use them in combination.

[Means for solving the problem]

The present inventors have made investigations so as to attain the purpose. Because the novel fungus Geotrichum candidum Dec 1 strain exerts wide decolorizing spectra over

various dyes, the inventors have made further investigations with their attention focused on the dye-degrading enzymes produced by the fungus. The inventors have successfully isolated and identified one of the enzymes, elucidated the gene encoding the enzyme and developed a mass expression system of the enzyme.

The invention according to claim 1 is a peroxidase derived from Geotrichum candidum Dec 1 strain (FERM P-15348), which has the following properties:

a) a property to degrade and decolorize dyes;

b) a molecular weight of 60 kDa, by the molecular weight assay using SDS-PAGE;

c) a molecular weight of 55 kDa, by the molecular weight assay using gel filtration; and

d) pI (isoelectric point) 3.8, by the assay by isoelectric focusing.

The invention according to claim 2 is the enzyme according to the claim 1, having the amino acid sequence of SQ ID NO. 7 in the sequence listing.

The invention according to claim 3 is the gene encoding the enzyme according to the claim 1, having the DNA sequence of SQ ID NO. 8 in the sequence listing.

The invention according to claim 4 is an expression plasmid vector comprising the coding gene according to the claim 3.

The invention according to claim 5 is a microorganism transfected with the expression plasmid vector according to the claim 4.

The invention according to claim 6 is a method for degrading and decolorizing dyes, which comprises using the enzyme according to the claim 1 or the microorganism according to the claim 5 for degrading and decolorizing dyes.

[Embodiment of the invention]

The invention is now described in detail hereinbelow.

The peroxidase of the invention according to claim 1 is derived from Geotrichum candidum Dec 1 strain. The inventors isolated and purified the enzyme as follows.

[Preparation of culture broth]

According to general methods, Geotrichum candidum Dec 1 strain (FERM P-15348) was cultured in a liquid culture medium. Any liquid culture medium of any composition can be used, as long as Geotrichum candidum Dec 1 strain can grow in the liquid culture medium. One preferable example is the potato dextrose culture medium (sometimes abbreviated as PD hereinbelow) manufactured by Difco, Co., Ltd. So as to promote the induction of the intended enzyme, further, dyes may be added to the culture medium.

The cultivating conditions of the fungal strain may satisfactorily be determined in light of the type of the culture medium used. When the PD culture medium is selected, for

example, the fungal strain is cultured at 15 to 37 °C, preferably at 30 °C for 3 to 8 days.

The culture broth thus recovered is subjected as a starting material for the purification of the dye-degrading enzyme to the following steps.

[Purification of dye-degrading enzyme]

Next, the dye-degrading enzyme is to be purified. The purification conditions are not specifically limited. For the purpose of the protection of the enzyme activity against inactivation, the culture broth is preferably handled at a low temperature, particularly in refrigerator.

Specifically, the microorganisms are first separated from the culture broth, from which the supernatant is recovered. In that case, separation processes such as filtration, centrifugation and membrane filtration may satisfactorily be used. Preferably, however, the microorganisms are removed via centrifugation, followed by filtration with glass filter. Contaminating polysaccharides are removed by performing the combination thereof, to recover a crude enzyme solution at a high purity.

Subsequently, the recovered crude enzyme solution may be subjected to isolation, by using the dye-degrading activity described below as a marker. Prior to such isolation, however, concentration and desalting may be carried out so as to readily enable the separation, to prepare a concentrated crude enzyme

solution.

Concentration may be done by methods for general use, for example ultra-filtration, salting-out, and evaporation. Preferably, concentration may be carried out by ultra-filtration. Additionally, desalting may be carried out by dialysis, ultra-filtration and electro-dialysis.

From the recovered concentrated crude enzyme solution can then be isolated the intended dye-degrading enzyme, by using the dye-degrading activity as the marker.

As the method therefor, ion exchange resin column chromatography, hydrophobic column chromatography, gel filtration column chromatography and the like may be used.

One of these column chromatography types or a combination of several types thereof may be used, to collect active fractions to isolate and purify the dye-degrading enzyme.

By the procedures, the inventors recovered the intended purified enzyme. The purified enzyme (205-fold active product) is at a single band by SDS-polyacrylamide gel electrophoresis (sometimes abbreviated as SDS-PAGE hereinbelow). The enzyme is the dye-degrading enzyme, peroxidase in the first aspect of the invention. The inventors designated the enzyme DyP.

[Properties of the purified dye-degrading enzyme DyP]

The properties of the inventive enzyme DyP purified by the procedures were measured according to the following principles.

First, the molecular weight was measured by SDS-PAGE and gel filtration chromatography.

For the measurement by SDS-PAGE, a commercially available molecular weight standard kit for electrophoresis may be used as the molecular weight standard.

One example thereof includes CONBITHEK manufactured by Boehringer Mannheim Yamanouchi, Co., Ltd. The kit comprises α-2-macroglobulin (molecular weight of 170 kDa), phosphorylase B (molecular weight of 97.4 kDa), glutamate dehydrogenase (molecular weight of 55.4 kDa), lactate dehydrogenase (molecular weight of 36.5 kDa), and trypsin inhibitor (molecular weight of 20.1 kDa).

As shown in Fig. 1 depicting the results of the measurement, the molecular weight of the inventive enzyme DyP is 60 kDa.

For the assay of the molecular weight of the enzyme by gel filtration chromatography, additionally, the inventors used Sephacryl S-200 column and the standard molecular weight protein (manufactured by BIO-RAD, CO., LTD.).

Consequently, the molecular weight of the inventive enzyme DyP was assayed as 55 kDa.

Then, the isoelectric point of the inventive enzyme was measured. The isoelectric point was measured by isoelectric focusing.

Consequently, the isoelectric point of the enzyme DyP was

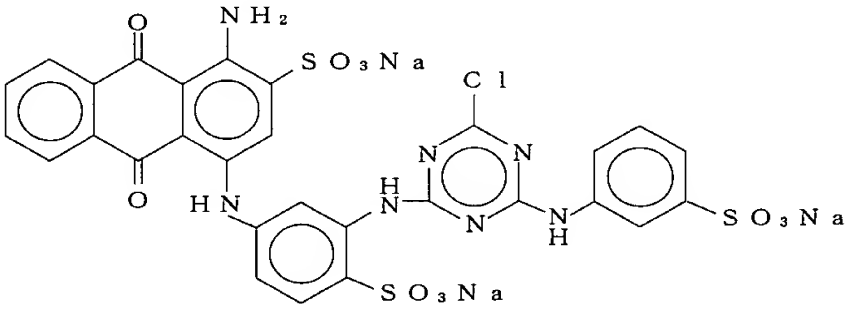
assayed as pI = 3.8, as shown in Fig. 2.

[Dye-degrading spectrum of dye-degrading enzyme DyP]

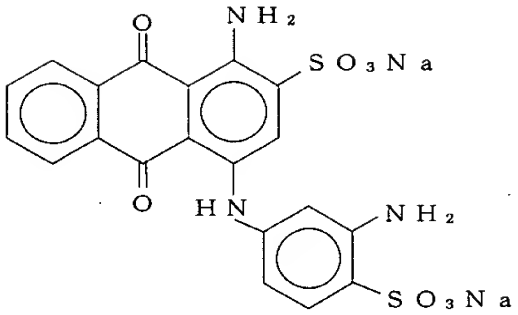
The dye-degrading enzyme DyP of the invention has an enzyme activity over azo type- and anthraquinone type- dyes, in particular, among dyes, and has an ability to degrade and decolorize these pigments.

The anthraquinone type dyes include for example Reactive blue 5, Reactive blue 19 and Reactive blue 114 (all manufactured by Nippon Kayaku Co., Ltd.);
1-amino-4-(3-amino-4-sodium-sulfonoanilino)-2-sodium anthraquinone sulfonate (sometimes abbreviated as AQ-1 hereinafter) and
1-amino-4-methylamino-2-sodium-anthraquinone sulfonate (sometimes abbreviated as AQ-2 hereinafter).

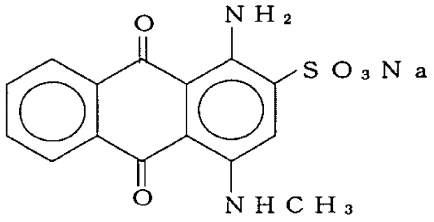
Herein, Reactive blue 5 is the compound represented by the following chemical formula.



AQ-1 is the compound represented by the following chemical formula.



AQ-2 is the compound represented by the following



Further, the azo type dyes include for example Reactive black 5, Reactive red 33, Reactive yellow 2 and Reactive blue 182 (all manufactured by Nippon Kayaku Co., Ltd.).

Other than the dyes, the dye-degrading enzyme DyP has an ability to degrade phenolic compounds such as 2,6-dimethoxyphenol and guaiacol, which are known as substrates for manganese peroxidase (sometimes abbreviated as MnP hereinafter). As shown in the following examples, however, no effect of manganese compounds added to the reaction solution on the promotion of the enzyme activity is observed.

Alternatively, no reaction of DyP with veratryl alcohol known as a substrate of lignin peroxidase (sometimes abbreviated as LiP hereinafter) is observed.

As described above, surprisingly, DyP exerts substrate

specificity different from those of MnP and LiP known so far. Thus, DyP can be said as a peroxidase differing from the known enzymes.

[Optimum reaction temperature of dye-degrading enzyme DyP]

The optimum reaction temperature of the inventive dye-degrading enzyme DyP is around 30 °C, as shown in Fig. 3. The enzyme DyP exerts stable dye-degrading activity within a temperature range of 15 °C to 35 °C. However, the enzyme activity rapidly decreases at a temperature above 35 °C.

[Temperature stability of dye-degrading enzyme DyP]

After the inventive dye-degrading enzyme DyP was stored at a fixed temperature for a fixed period of time, the ratio of the remaining activity was subsequently evaluated. Specifically, a solution of the dye-degrading enzyme DyP in 25 mM citrate buffer was stored at 30 °C or 40 °C for 14 days.

Consequently, the remaining enzyme activity of DyP was at 63 % when DyP was stored at 30 °C; and the activity was at 41 % when DyP was stored at 40 °C.

So as to compare the temperature stability with those of other peroxidases, solutions of individual enzymes in 25 mM citrate buffer were stored at 60 °C for 3 hours, by using a commercially available horse radish peroxidase (manufactured by Wako Chemical Co., Ltd.; sometimes abbreviated as HRP hereinafter) as the control. Subsequently, the remaining enzyme activities were compared to each other.

Consequently, it was shown that 65 % of the activity of the inventive dye-degrading enzyme DyP remained, but only 10 % of the activity of HRP remained.

The results show that the dye-degrading enzyme DyP has greater thermal stability, compared with currently known peroxidases.

The enzyme characteristics of the novel dye-degrading enzyme DyP in accordance with the invention are described hereinabove.

The dye-degrading enzyme DyP of the invention exerts a wider range of dye-degrading activity, compared with any of currently reported dye-degrading enzymes, and has also prominent enzyme stability, as apparently shown in the enzyme characteristics.

In the sixth aspect of the invention, hence, the use of the dye-degrading enzyme DyP enables efficient degradation and decolorization of such dyes.

So as to raise the industrial applicability of the dye-degrading enzyme DyP, essentially, efficient degradation of wastewater containing dyes and dyes should be attained economically.

One example includes a method using DyP after immobilization. The method comprises immobilizing DyP through adsorption or covalent bonding on immobilization carriers, such as ion exchange resin, synthetic polymer gel, naturally

originated active charcoal and zeolite, and using the resulting DyP as bioreactor. For creating highly active bioreactor, the method is more useful than the method using the microorganism per se after immobilization.

As a means for more economically producing the enzyme, the gene encoding the intended enzyme is isolated, which is then introduced in a host microorganism capable of expressing the enzyme at a mass scale, so that DyP at a higher purity can be recovered more efficiently in a more stable manner.

Compared with the case of using the microorganism per se, a combination thereof enables the preparation of a bioreactor with a far more excellent cost performance.

[Schema of isolation of gene encoding dye-degrading enzyme DyP]

From the above respects, the inventors carried out the following procedures so as to obtain the genetic information of the DyP of the invention.

The concrete method for isolating the gene is schematically described below.

First, partial hydrolysis of the inventive dye-degrading enzyme DyP purified by the aforementioned method was carried out by allowing trypsin (manufactured by Wako Chemical Co., Ltd.) to react with the enzyme.

The resulting five types of the partially hydrolyzed fragments were purified. Thereafter the amino acid sequences of the individual fragments were determined, to synthetically

prepare the coding gene corresponding to each of the amino acid sequences.

Subsequently, PCR-amplified gene was recovered by PCR using the resulting coding gene as a primer and the cDNA derived from Geotrichum candidum as a template.

The resulting amplified gene was labeled by using the DIG labeling detection kit (manufactured by Boehringer Mannheim, Co., Ltd.).

By general methods using the labeled amplified gene as a probe, plaque hybridization with the Geotrichum candidum Dec 1-derived cDNA library prepared by using lambda phage λgt10, was performed.

From some hybridized colonies thus recovered were cut out the intended genes, which were then integrated into pUC18 plasmid, for subsequent sequencing. This was used as template for the following PCR.

[Determination of partial amino acid sequence of dye-degrading enzyme DyP]

So as to prepare a primer for the gene encoding DyP, DyP was purified.

Purification of DyP can be performed by usual methods. The methods include for example a purification method comprising electroblotting from SDS-PAGE gel and a purification method by high-performance liquid chromatography (HPLC).

After the purified DyP was denatured by ordinary methods, partial hydrolysis thereof using trypsin was performed. Partially digested peptides thus formed were fractionated by HPLC. Consequently, five fragments were recovered. The amino acid sequence of each of the fragments was determined by the Edman method with a protein sequencer. Among the amino acid sequences of the resulting five fragments, the first sequence was Trp Lys. The amino acid sequences of the second and thereafter are shown in the sequence listing, where the second is shown in SQ ID NO. 1; the third is shown in SQ ID NO. 2; the fourth is shown in SQ ID NO. 3 and the fifth is shown in SQ ID NO. 4.

Among these amino acid sequences, a partial sequence (SQ ID NO. 5) of SQ ID NO. 3 and a partial sequence (SQ ID NO. 6) of SQ ID NO. 4 were selected as PCR primers.

[Probe preparation]

DNA encoding the two types of amino acid sequences was synthetically prepared by the following method.

By PCR using the resulting primer genes and the cDNA derived from Geotrichum candidum Dec 1 as PCR template, a first-stage gene amplification was practiced. Consequently, new 200-bp primers corresponding to the two primers were recovered.

Both the termini of the primers were subjected to T4 DNA polymerase treatment, to synthetically prepare

plasmid-ligation sites. Then, the primers were ligated to the Hinc II site of pUC 18 as E. coli expression vector, to recover a recombinant plasmid.

The recombinant plasmid was amplified, by using E. coli JM 109 strain. From the resulting plasmid was cut out the coding gene. By a second PCR, the resulting DNA was sequenced (see the positions 1012 to 1181 of SQ ID NO. 8 in the sequence listing).

[Cloning of gene dyP encoding dye-degrading enzyme DyP]

From the Geotrichum candidum Dec 1 strain cultured separately was prepared RNA according to usual methods. From the resulting RNA was purified poly(A)$^+$RNA. Subsequently, the recovered poly(A)$^+$RNA was used to prepare cDNA with a cDNA kit (manufactured by TaKaRa).

After the recovered cDNA was subjected to ligation with T4 polynucleotide kinase kit, DNA of 1,200 to 2,000 bp was fractionated by electrophoresis.

Further, the DNA was inserted in the Eco RI site of lambda phage λgt10, for packaging into the λ phage. The recovered phage was used for infection of E. coli.

Colonies hybridizing with the labeled probe previously prepared were screened. As a result, 11 candidates were obtained.

The results of the measurement described above indicate that DyP of the invention has a molecular weight of 60 kDa at

a sugar chain content of 17 %, so the primary amino acid sequence is estimated to be 49.8 kDa. Additionally, the open reading frame of the gene encoding DyP is estimated to comprise 460 amino acids, namely 1380 bp.

Independently using the coding genes of the recovered 11 candidates, PCR was carried out again to evaluate the fragment size of the inserted cDNA. In other words, genes in the proximity of 1380 bp were screened.

Consequently, clone 92 carrying the cDNA of a 1600-bp size was recovered. The cDNA was cut out with Bam HI from the recombinant plasmid, which was then integrated in pUC18. The resulting plasmid was designated pB92. It was verified that the clone 92 had a dye-degrading activity based on the dye-degrading enzyme DyP.

[DNA sequence of pB92 gene]

pB92 was sequenced with DNA sequencer. Consequently, it was found that the open reading frame of pB92 comprised 498 amino acids, namely 1494 bp and had a molecular weight of 53,306.

This indicates that pB92 carries the DyP gene. The nucleotide sequence of the DyP gene and the amino acid sequence of DyP, carried in pB92, are shown as SQ ID NOS. 7 and 8, respectively. In other words, DyP having the amino acid sequence described as SQ ID NO. 7 in the sequence listing is the enzyme described in the second aspect of the invention,

while the gene having the nucleotide sequence described as SQ ID NO. 8 in the sequence listing is the gene in the third aspect of the invention.

Herein, the gene in the third aspect of invention (see SQ ID NO. 8 in the sequence listing) when modified with deletion, substitution, addition and the like in a part of the sequence is also encompassed within the scope of the invention, as long as the resulting modified gene has the same effects as those of the gene of the invention.

Further, pB92 as the plasmid vector carrying these genes is described in the fourth aspect of the invention.

Still further, a transformant recovered by transfecting E. coli with pB92 is described in the fifth aspect of the invention. When the transformant is used, the dye-degrading enzyme DyP of the invention can efficiently be produced.

[Examples]

The invention will now be described more specifically in examples hereinbelow. However, the invention is not limited to the examples.

Example 1

(Purification and properties of dye-degrading enzyme DyP)

[Purification of dye-degrading enzyme DyP]

150 mL of a PD culture medium (potato-dextrose culture medium, manufactured by Difco, Co., Ltd.) was placed in a 500-mL Erlenmeyer flask, into which 5 ml of the spore suspension of

Geotrichum candidum Dec 1 (FERM P-15348) strain was inoculated. Then, culturing was started. Culturing was continued at 30 °C and 120 rpm for 6 days.

After culturing, the culture broth was cooled to 4 °C and centrifuged at 7,200 × g for 20 minutes. 4,380 mL of the resulting supernatant was used for the following procedures.

The supernatant was filtered through a glass filter (GC50, manufactured by Toyo Roshi Co., Ltd.), to remove the polysaccharide contained therein.

Then, the filtrate was subjected to ultrafiltration on an ultrafiltration membrane (YM10) manufactured by Amicon, Co., Ltd., to concentrate the filtrate to 60 mL. The concentrate was dialyzed against 25 mM piperazine buffer (pH 5.5) and was then concentrated to 17.2 mL, by using Centriprep10 manufactured by Amicon, Co., Ltd.

The concentrate of 17.2 mL was charged on Super Q 650 M column of 2.8 × 6.0 cm (manufactured by Tosoh Co., Ltd.), which was preliminarily equilibrated with 25 mM piperazine buffer (pH 5.5). Subsequently, the column was rinsed with 200 mL of the same buffer, followed by elution on a linear gradient of 0 to 0.4 M.

Fractions with dye-degrading activity were collected and concentrated to 2.8 mL, by using Centriprep10 manufactured by Amicon, Co., Ltd. The concentrate was charged on Butyl Toyopearl of 1.6 × 6.5 cm (manufactured by Tosoh Co., Ltd.),

which was preliminarily equilibrated with 25 mM citrate buffer (pH 5.5) and 0.8 M ammonium sulfate. Subsequently, the column was rinsed with 50 mL of the same buffer, followed by elution on a linear gradient of ammonium sulfate from 0.8 M to 0, to collect a fraction with the dye-degrading activity, which was defined DyP.

DyP was dialyzed against 25 mM citrate buffer, to recover purified DyP at 1.5 mg. The purified DyP solution was stored at 4 °C.

[Properties of dye-degrading enzyme]

The molecular weight and isoelectric point of the dye-degrading enzyme DyP recovered by the above procedures were measured.

The molecular weight was determined by SDS-PAGE electrophoresis and gel filtration method.

For SDS-PAGE electrophoresis, 10 % polyacrylamide gel and an electrophoresis apparatus of AE-6440 manufactured by Atto Co., Ltd. were used. As the molecular weight control, further, Combithek manufactured by Boehringer Mannheim Yamanouchi, Co., Ltd. was used.

Consequently, the molecular weight of DyP was assayed as 60 kDa.

For gel filtration, alternatively, Sephacryl S-200 column of 3.1 × 95 cm after equilibration with 25 mM citrate buffer (pH 5.0) was used, together with the standard protein

kit manufactured by BIO-RAD Co., Ltd.

Consequently, the molecular weight of DyP was assayed as 55 kDa.

For measurement of isoelectric focusing, a low-pI calibration kit of Multiphor II 2-D for pH 2.5 to pH 6.5, manufactured by Pharmacia, Co. was used. Consequently, the isoelectric point of DyP was assayed as 3.8.

[Assay of dye-degrading activity]

The dye-degrading spectrum of the purified DyP was examined for nine types of dyes and three model compounds. The activity of the purified DyP to degrade these dyes or model compounds was assayed by measuring the degradation rates.

As the dyes, use was made of Reactive blue 5, 19 and 114; AQ-1 and AQ-2; Reactive black 5, Reactive red 33, Reactive yellow 2, and Reactive blue 182.

As the model compounds, additionally, use was made of 2,6-dimethoxyphenol, guaiacol and veratryl alcohol.

The dye-degrading activity was measured as follows.

0.2 to 0.4 mM aqueous hydrogen peroxide was added to a mixture solution of 3 mL of 25 mM citrate buffer (adjusted to the optimum pH for the degradation of each of the dyes) containing each dye at a fixed pH (30 to 120 ppm) and 1 mL of 1.86 nM DyP solution, to initiate the enzyme reaction. Reaction was performed at 30 °C for a fixed period of time, to assay the reaction rate.

1 U of the dye-degrading activity was defined as the activity to decolorize 1 μmole RB5 or AQ-2 for one minute. The results are shown in Table 1.

[Table 1]

Table 1 (DyP activity to degrade each dye and model compound)

Color index	Chromogen	λmax	Optimum pH	Initial concentration (ppm)	Decolorizing activity (ppm/min)
Reactive blue 5	AQ	600	3.2	100	19.8
Reactive blue 19	AQ	590	3.2	70	13.1
Reactive blue 114	AQ	620	4.0	100	7.8
AQ-1	AQ	600	3.2	60	5.4
AQ-2	AQ	635	3.0	50	19.5
Reactive black 5	AZ	598	3.2	30	0.1
Reactive red 33	AZ	500	3.2	50	0.4
Reactive yellow 2	AZ	390	3.2	100	0.5
Reactive blue 182	AZ	610	4.0	120	20.9

As to 2,6-dimethoxyphenol used as a model compound, alternatively, absorbance at 470 nm was colorimetrically measured, which emerged via oxidation.

That is to say, a mixture solution of 2.79 nM DyP and 0.2 mM 2,6-dimethoxyphenol was reacted with 25 mM citrate buffer (pH 4.5) containing 0.2 mM hydrogen peroxide.

As to guaiacol, 1 mM guaiacol was used in place of 0.2 mM 2,6-dimethoxyphenol, for absorbance measurement at 465 nm.

The results about the model compounds are shown in Table

2.

[Table 2]

Table 2 (DyP activity to degrade model compounds)

Compound	Group	pH	Initial concentration (mM)	Oxidation rate (ΔOD/min)
2,6-Dimethoxyphenol	phenolic	4.5	0.2	0.29
Guaiacol	phenolic	4.0	1.0	0.29
Veratryl alcohol	nonphenolic	-	0.5	ND

The results in Table 1 indicate those described below.

The dye-degrading enzyme DyP exerts a high activity to degrade the anthraquinone type pigments. Specifically, the enzyme exerts an excellent degradation activity over Reactive blue 5, Reactive blue 19 and AQ-2, so the enzyme can efficiently degrade these pigments.

Additionally, the enzyme exerts an activity to degrade the azo type pigments. The enzyme efficiently degraded Reactive blue 182, in particular. The enzyme has an ability to degrade other azo type pigments, Reactive black 5, Reactive red 33 and Reactive yellow 2.

This apparently demonstrates that DyP has an action to degrade anthraquinone type pigments and azo type pigments.

Alternatively, the DyP activity over the model compounds is as follows, on the basis of the results in Table 2.

First, 2,6-dimethoxyphenol and guaiacol having phenolic hydroxyl group could efficiently be degraded by DyP.

Alternatively, DyP could never degrade veratryl alcohol known as a substrate of lignin peroxidase.

This apparently indicates that DyP has a specifically high enzyme activity over the compounds having phenolic hydroxyl group.

[Optimum temperature of dye-degrading enzyme DyP]

The optimum temperature of DyP was determined by examining the decolorizing (degrading) activity of Reactive blue 5 at a fixed temperature. The results are shown in Fig. 3.

Fig. 3 indicates that DyP exerts a high peroxidase activity within a range of 20 to 35 °C, and also indicates that the optimum temperature is 30 °C.

Example 2

[Effect of metal ion on the activity of dye-degrading enzyme DyP]

Each 5 mM ions of calcium, zinc, copper (divalent), potassium, iron (divalent) and sodium were concurrently present in a reaction solution comprising DyP and 100 ppm Reactive blue 5, so as to examine the effects of these metal ions on the relative activity of the DyP enzyme.

The results are shown in Table 3.

[Table 3]

Table 3 (Influence of metallic cation on DyP activity to degrade dyestuff)

Metal ion	Concentration (mM)	Specific activity (%)
No addition	-	100
Ca^{++}	5	81
Zn^{++}	5	69
Cu^{++}	5	75
K^{+}	5	81
Na^{+}	5	81
Fe^{++}	0.2	50

Table 3 shows that the enzyme exhibited a relative activity of about 80 %, when the ions were added, compared with the case of no addition. Particularly, the divalent iron ion concurrently present at 0.2 mM inhibited the activity at 50 %.

This indicates that the concurrent presence of the metal ions affects adversely the enzyme activity of DyP.

Example 3

(Determination of gene encoding DyP and the amino acid sequence thereof)

[Determination of partial amino acid sequence of dye-degrading enzyme DyP]

According to the Laemmli method (Laemmli, U.K. Nature (London), 227, 680-685 (1970)), Geotrichum candidum Dec 1 strain was subjected to SDS-PAGE, to separate purified DyP.

Subsequently, the DyP was electroblotted on polyvinyl difluoride (sometimes abbreviated as PVDF hereinafter) membrane according to the Towbin method (Towbin, H., Staehelin, T., and Gordon, J. Proc. Natl. Acad. Sci. USA, 76, 4350-4354 (1979)).

The PVDF membrane was treated with Coomassie Brilliant Blue (CBB-250), from which was then cut out solely the membrane corresponding to the stained band portion of the DyP. The band was then transferred in a 1.5-mL test tube. 50 μL methanol was added to the test tube, followed by addition of 200 μL reductive buffer (buffer, pH 8.5, containing 8 M guanidine hydrochloride salt, 0.5 M Tris buffer, 0.3 % EDTA-2Na and 5 % acetonitrile), for gradual shaking, from which the reductive buffer was removed.

Then, 50 μL reductive buffer containing 1 mg dithiothreitol was added onto the protein on the PVDF membrane, which was then left at 25 °C for one hour. After the PVDF membrane was transferred in a 200-mL conical beaker, followed by sequential rinsing individually with 100 mL water for 5 minutes, 100 mL 2 % acetonitrile for 5 minutes and 100 mL 0.1 % SDS for 5 minutes.

Thereafter, the PVDF membrane was transferred into a fresh 1.5-mL test tube, followed by addition of 500 μL polyvinylpyrrolidone PVP-40 (sometimes abbreviated as PVP-40 hereinafter) containing 1 mg methionine according to the Iwamatsu method (Iwamatsu, A. Electrophoresis, 13, 142-147 (1992)), and the resulting mixture was left to stand at ambient temperature for 30 minutes.

After the PVDF membrane was additionally rinsed with 100 mL 10 % acetonitrile solution, it was rinsed three times with

500 μL of degradation buffer (100 mM ammonium bicarbonate, 10 mM calcium chloride, pH 7.8). Then the rinse solutions were discarded. Continuously, 500 μL of the same degradation buffer except for the content of 1 pmol trypsin was added, and then enzymatic reaction was carried out at 25 °C for 12 hours.

After the oligopeptide eluted from the PVDF membrane into the reaction solution was freeze-dried, the freeze-dried oligopeptide was then dissolved in 100 μL degradation buffer, followed by elution on a 0-50 % linear gradient (100 minutes, 0.8 mL/min) of isopropyl alcohol-acetonitrile (7:3 v/v) containing 0.02 % trifluoroacetic acid by HPLC (column: Capcell-Pak C-18, 4.6 × 150 mm), to fractionate individual fractions.

The partially degraded peptides fractionated and purified were determined of their primary structures with a protein sequence system (Shimadzu, PPSQ-21).

[Preparation of cDNA library]

For the purpose of RNA extraction, the culture broth of Geotrichum candidum Dec 1 strain was subjected to centrifugation, to separate 25 mL of hypha of the strain. This was placed in a centrifuge tube, followed by addition of liquid nitrogen to freeze the hypha, and then it was freeze-dried for 12 hours to recover a powder.

Over the resulting powder of the hypha was again poured liquid nitrogen, to pulverize the hypha, to which was then added

10 mL of a guanidium solution (containing 4 M guanidium isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.1 mM DTT and 0.5 % N-lauroylsarcosine), for homogenization. This was then centrifuged (1,500 × g) to obtain the supernatant.

The resulting RNA was separated by cesium chloride ultra-centrifugation method (Ullrich), followed by fractionation of poly(A)$^+$ RNA using oligo(dT) cellulose column. The resulting poly(A)$^+$ RNA was used to synthetically prepare cDNA with a cDNA synthesis kit (TaKaRa; Gulbler-Hoffman).

Into the resulting cDNA was inserted an adaptor (EcoRI-NotI-BamHI), by using a DNA ligation kit. Both the ligated termini were phosphorylated with T4 polynucleotide kinase, to separate cDNA by agarose electrophoresis.

Further, 1200- to 2000-bp cDNA corresponding to the molecular weight of DyP was separated among the resulting cDNAs, to which was then added 8 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA-2Na) to elute the cDNA.

The recovered cDNA fragment was ligated in the EcoRI site of λ phage. This was packaged into λ phage with Gigapack Gold Packaging Extract (Stratagene, La Jolla Calif., USA).

After *E. coli* NM514 strain was infected with lambda phage λgt 10 at 37 °C for 15 minutes, the infected strain was overlaid on LB agar medium (bacto-tryptone, 0.5 % bacto-yeast extract, 1 % sodium chloride, 1.5 % agar/1,000 mL), by using 0.7 % agar. The plate was cultured at 37 °C for 12 hours.

[Sequencing of coding gene of dye-degrading enzyme DyP]

Colonies hybridizing with the preliminarily prepared labeled probe were screened. That is to say, by plaque hybridization of the colonies, 11 candidates were consequently selected from the positive cDNA library and DNAs recovered by PCR.

These were ligated with pUC18 plasmid via T4 DNA ligase, for amplification in E. coli JM109 strain.

Because DyP has a molecular weight of 60 kDa at a sugar chain content of 17 %, on the basis of the results of the measurement of the DyP properties, the primary amino acid sequence is estimated to be of 49.8 kDa. Additionally, it is estimated that the open reading frame of the DyP coding gene will comprise 460 amino acids, namely 1380 bp.

Then, the coding genes of the resulting 11 candidates were used for PCR again. Among the inserted cDNAs, a gene around 1380 bp was screened.

Consequently, clone 92 carrying the cDNA of a 1600-bp size was obtained.

By using BamHI, the cDNA was cleaved out of the recombinant plasmid, which was then inserted in pUC18. The resulting plasmid was designated pB92.

Subsequently, the plasmid DNA was prepared by the alkali extraction method. Both the resulting strands were analyzed and sequenced by a DNA sequencer (Model 4000L, Li-Cor Inc.,

Lincoln, Neb., USA).

The open reading frame of the pB92 thus recovered comprises 1494 bp, namely 498 amino acids (see SQ ID NO. 7 in the sequence listing). Thus, the molecular weight estimated from the number of the amino acids was 53,306. This indicates that the pB92 carries the DyP gene.

Furthermore, pB92 was transfected into E. coli.

[Effect of the invention]

In accordance with the invention, the peroxidase with high degradation activity of a wide range of dye types as well as the method for degrading the dye types by using the enzyme can be provided. Additionally, the invention provides the genetic information of the enzyme and can supply the enzyme at a large scale on the basis of the information. Thus, the enzyme can be applied to the treatment of wastewater containing dyes and the like, in the fields of staining industry and the like.

Accordingly, the immobilization of the enzyme as a dye-degrading enzyme can raise the industrial applicability thereof as a bioreactor with higher activity.

[Sequence Listing]

SEQUENCE LISTING

<110> Meiji Seika Kaisha Ltd.

<120> Novel enzyme with decolorizing activity and method for decolorizing dyes by using the same

<130> P111024K

<160> 8

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<211> 6
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1 5

<210> 2
<211> 8
<212> PRT
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1 5

<210> 3
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Thr Ala Gln Asp Arg
20

<210> 4

<211> 21
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1 5

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<400> 6
Pro Thr Ala Asp Asp Pro
1 5
<210> 7
<211> 498
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<400> 7

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            20                  25                  30
Thr Pro Leu Leu Thr Asn Phe Pro Gly Gln Ala Pro Leu Pro Thr Leu
        35                  40                  45
Thr Gln His Thr Thr Glu Ser Gly Ala Asn Asp Thr Ile Leu Pro Leu
    50                  55                  60
Asn Asn Ile Gln Gly Asp Ile Leu Val Gly Met Lys Lys Gln Lys Glu
65                  70                  75                  80
Arg Phe Val Phe Phe Gln Val Asn Asp Ala Thr Ser Phe Lys Thr Ala
                85                  90                  95
Leu Lys Thr Tyr Val Pro Glu Arg Ile Thr Ser Ala Ala Ile Leu Ile
            100                 105                 110
Ser Asp Pro Ser Gln Gln Pro Leu Ala Phe Val Asn Leu Gly Phe Ser
        115                 120                 125
Asn Thr Gly Leu Gln Ala Leu Gly Ile Thr Asp Asp Leu Gly Asp Ala
    130                 135                 140
Gln Phe Pro Asp Gly Gln Phe Ala Asp Ala Ala Asn Leu Gly Asp Asp
145                 150                 155                 160
Leu Ser Gln Trp Val Ala Pro Phe Thr Gly Thr Thr Ile His Gly Val
                165                 170                 175
Phe Leu Ile Gly Ser Asp Gln Asp Asp Phe Leu Asp Gln Phe Thr Asp
            180                 185                 190
Asp Ile Ser Ser Thr Phe Gly Ser Ser Ile Thr Gln Val Gln Ala Leu
        195                 200                 205
Ser Gly Ser Ala Arg Pro Gly Asp Gln Ala Gly His Glu His Phe Gly
    210                 215                 220
Phe Leu Asp Gly Ile Ser Gln Pro Ser Val Thr Gly Trp Glu Thr Thr
225                 230                 235                 240
Val Phe Pro Gly Gln Ala Val Val Pro Pro Gly Ile Ile Leu Thr Gly
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245 250 255

Arg Asp Gly Asp Thr Gly Thr Arg Pro Ser Trp Ala Leu Asp Gly Ser

260 265 270

Phe Met Ala Phe Arg His Phe Gln Gln Lys Val Pro Glu Phe Asn Ala

275 280 285

Tyr Thr Leu Ala Asn Ala Ile Pro Ala Asn Ser Ala Gly Asn Leu Thr

290 295 300

Gln Gln Glu Gly Ala Glu Phe Leu Gly Ala Arg Met Phe Gly Arg Trp

305 310 315 320

Lys Ser Gly Ala Pro Ile Asp Leu Ala Pro Thr Ala Asp Asp Pro Ala

325 330 335

Leu Gly Ala Asp Pro Gln Arg Asn Asn Asn Phe Asp Tyr Ser Asp Thr

340 345 350

Leu Thr Asp Glu Thr Arg Cys Pro Phe Gly Ala His Val Arg Lys Thr

355 360 365

Asn Pro Arg Gln Asp Leu Gly Gly Pro Val Asp Thr Phe His Ala Met

370 375 380

Arg Ser Ser Ile Pro Tyr Gly Pro Glu Thr Ser Asp Ala Glu Leu Ala

385 390 395 400

Ser Gly Val Thr Ala Gln Asp Arg Gly Leu Leu Phe Val Glu Tyr Gln

405 410 415

Ser Ile Ile Gly Asn Gly Phe Arg Phe Gln Gln Ile Asn Trp Ala Asn

420 425 430

Asn Ala Asn Phe Pro Phe Ser Lys Pro Ile Thr Pro Gly Ile Glu Pro

435 440 445

Ile Ile Gly Gln Thr Thr Pro Arg Thr Val Gly Gly Leu Asp Pro Leu

450 455 460

Asn Gln Asn Glu Thr Phe Thr Val Pro Leu Phe Val Ile Pro Lys Gly

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Gly Glu Tyr Phe Phe Leu Pro Ser Ile Ser Ala Leu Thr Ala Thr Ile

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Ala Ala

498

<210> 8

<211> 1494

<212> DNA

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aca ccc ctc ctc act aat ttt ccg gga caa gcc ccg ctg ccg act cta 144
acg cag cat acg act gag agc ggg gcc aac gat aca att ctg ccc ctg 192
aac aac ata caa ggc gac att ttg gtt ggc atg aag aaa cag aag gaa 240
cgc ttc gtc ttt ttc caa gtc aat gac gca acc tcg ttc aag acg gcg 288
ttg aag acc tac gtg cct gag cgc atc acg tcg gcg gcg att ttg att 336
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aac aca ggc ctc cag gcg ctt gga att acc gac gat ctg ggt gat gca 432
caa ttc cca gat ggt cag ttc gca gac gcc gca aac ctc ggg gac gac 480
ctc agc caa tgg gtg gcg cct ttt act ggt acc acc atc cat ggt gtc 528
ttt ctg att ggt agc gac cag gac gac ttc ttg gat cag ttc acg gat 576
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gct gct 1494

[Brief description of the drawing]

[Fig. 1] depicts the results of the SDS electrophoresis of the enzyme DyP of the invention.

[Description of numerals] In the figure, the left numerical figures show molecular weight, while the upper numerical figures 1 to 5 independently represent the molecular weight marker, crude enzyme solution, the enzyme solution after ion exchange chromatography, the enzyme solution after hydrophobic chromatography and the enzyme solution after ion exchange chromatography, in this order.

[Fig. 2] depicts the results of the isoelectric focusing of the enzyme DyP of the invention.

[Description of numerals] In the figure, the right numerical figures show isoelectric points (pI), while the upper numerical figures 1 to 3 independently represent crude enzyme solution, purified DyP and the isoelectric point marker, in this order.

[Fig. 3] is a graph depicting the relation between the enzyme activity of the inventive enzyme DyP and temperature.

Fig. 1

Fig. 2

Fig.3

[Name of document] Abstract

[Abstract]

[Problem] Provided are development of an enzyme applicable to more efficient treatment of wastewater containing dyes and a method for degrading and decolorizing dyes by using the enzyme as well as realization of mass supply of the enzyme by isolation and purification of said dye-degrading enzyme both to elucidate its genetic construction and to obtain the genetic information of said enzyme.

[Means for solution] (1) a peroxidase enzyme derived from Geotrichum candidum Dec 1 (FERM P-15348), which was specified with a molecular weight and pI (isoelectric point) and has a property to degrade and decolorize dyes; (2) said enzyme having the amino acid sequence of SQ ID NO. 7 in the sequence listing; (3) a gene encoding the above enzyme having the DNA sequence of SQ ID NO. 8 in the sequence listing.; (4) an expression plasmid vector comprising said gene; (5) a microorganism transfected with said expression plasmid vector; and (6) a method for degrading and decolorizing dyes, comprising using the above enzyme or the above microorganism for degrading and decolorizing dyes.

[Selected drawing] None